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L3: Entry 1 of 1 File: USPT Jul 3, 2001

DOCUMENT-IDENTIFIER: US 6255055 B1
TITLE: c-myc coding region determinant-binding protein (CRD-BP) and its nucleic acid sequence

US Patent No. (1):
6255055

Drawing Description Text (4):
FIG. 3. Immunoblotting assay showing co-migration of recombinant and cell derived CRD-BP. Ribosomal salt wash (RSW) was prepared from K562 and NIH/3T3 cell polysomes and from polysomes isolated from reticulocyte transcription/translation reactions programmed with CRD-BP DNA or with vector DNA. Approximately 7.5.times.10.sup.5 cell equivalents of K562 or NIH/3T3 RSW or 3% of the RSW recovered from a 50 .mu.l translation reaction were electrophoresed in a 10% SDS-PAG and transferred to a membrane, which was incubated with anti-CRD-BP IgY antibody and then with HRP-conjugated anti-IgY antibody. The signal was developed with Supersignal chemiluminescent reagents. The locations of the CRD-BP and a cross-reacting protein (p85) are indicated. The locations of prestained molecular mass markers are shown on the right in kDa.

Drawing Description Text (5):
FIG. 4. Gel retardation assay showing specific binding of recombinant CRD BP to c-myc CRD RNA. (A) RSW was prepared from K562 cell polysomes and from transcription/translation reactions programmed with CRD-BP cDNA, luciferase cDNA (Luc), or vector DNA. Equivalent volumes (2 .mu.l) of each RSW were incubated with 50,000 cpm of synthetic c-myc CRD .sup.32 P-RNA. RNA/protein complexes were separated from free (unbound) probe by electrophoresis in a 6% nondenaturing PAG. "None" indicates a gel retardation reaction to which no protein was added. The positions of CRD-BP/CRD complexes (Bound) and of unbound (Free) RNA are indicated on the left. (B) Competition assay. The indicated RSW was incubated with c-myc CRD .sup.32 P-RNA in the presence or absence of buffer (None) or a 200-fold molar excess of unlabeled synthetic c-myc CRD RNA or .beta.-Globin RNA. RNA/protein complexes were then separated in a 6% nondenaturing PAG. The positions of CRD-BP/CRD complexes (Bound) and of unbound (Free) RNA are indicated on the left.

Detailed Description Text (55):
In vitro translation of mouse CRD-BP. A portion of the mouse CRD-BP cDNA was subcloned into pSPUTK (Stratagene, La Jolla, Calif.) to create the translation clone pSPUTK-CRD-BP as follows: A single base mutation (underlined) was made in the 5' primer (5.degree. CGCACCGCCACCATGGACAAGCTTTACATCGG-3') (SEQ ID NO:43) to generate an NcoI site for subcloning. The mutation changes an asparagine to an aspartic acid. The 3' primer (5'-ACTGGGATCTGACCCATCCT-3') (SEQ ID NO:44) was from the CRD-BP 3'-UTR. Conditions were 1 cycle of 94.degree. C. for 1 minute, followed by 25 cycles of 94.degree. C. for 30 seconds, 55.degree. C. for 30 seconds, 68.degree. C. for 3 minutes. pSPUTK-CRD-BP, pSPUTK-Luciferase, or pSPUTK vector templates were transcribed and translated using the TnT.RTM. Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions.

Detailed Description Text (65):
Comparison of in vitro synthesized CRD-BP with cell-derived CRD BP. To determine whether our murine cDNA clone encoded full-length CRD-BP with the expected

properties of a c-myc mRNA-binding protein, we synthesized the protein in vitro and analyzed it by immunoblotting and gel retardation assays. Reticulocyte transcription/translation reactions were programmed with CRD-BP cDNA subcloned into a pSPUTK vector. The CRD-BP sequences in the subclone began with the AUG denoted as the translation start site in FIG. 1. This subclone did not contain the upstream, in-frame AUG. The translation extract was fractionated by SDS-PAGE and analyzed by immunoblotting with anti-CRD-BP antibody. A protein of .about.68 kDa from the cDNA translation was recognized by anti-CRD-BP antibody and migrated close to the positions of authentic CRD-BP from human (K562) and mouse (NIH/3T3) cells (FIG. 3, lanes 1-3). An immunoreactive band was not observed in control lanes containing extract programmed with the pSPUTK vector (FIG. 3, lane 4) or with luciferase cDNA (data not shown), indicating that the antibody specifically detected CRD-BP and not an endogenous reticulocyte protein. Therefore, our cDNA encodes CRD-BP. The cross-reacting band (p85) seen in the K562 and NIH/3T3 RSW lanes is a protein observed previously (32). Its identity and function are unknown. p85 does not bind c-myc CRD RNA (32), and it localizes to different subcellular fractions when compared to CRD-BP (see below).

Detailed Description Text (67):
An RNA/protein complex was not observed with protein from the luciferase (Luc), Vector, or no mRNA (None) control reactions (FIG. 4A, lanes 3-5). Therefore, in vitro synthesized CRD-BP, like its cell-derived counterpart, associates with c-myc CRD RNA in vitro.

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Detailed Description Text (12):
One typical way to obtain a CRD-BP antibody would be to make large amounts of recombinant CRD-BP in either bacterial cells, yeast cells or baculovirus-infected insect cells. This protein is then injected into rabbits, sheep or goats to make a polyclonal antibody. Epitope-specific antibodies can also be made by using synthetic peptides (8-15 amino acids) as the immunogen. These are routine techniques known to those of skill in the art.

Detailed Description Text (20):
i. Two antibody sandwich assay: A monoclonal antibody recognizing one CRD-BP epitope is bound to a solid support such as a microtiter well. The sandwich assay would also work with two polyclonal antibodies, as long as each antibody was against a different epitope in the CRD-BP. An extract of the tissue is added, and CRD-BP in the extract is permitted to bind to the antibody. Then a second monoclonal recognizing a different CRD-BP epitope is added. The second antibody can be labeled with .sup.125 I or .sup.3 H. Then, the amount of labeled antibody bound will provide a measure of the amount of CRD-BP attached to the first antibody.

Detailed Description Text (23):
Prokipcak, et al. (ref. 31) discloses one method of purification of CRD-BP. We also envision an easier purification scheme that exploits added epitopes. Instead of making unmodified CRD-BP in bacteria, yeast, or baculovirus-infected cells, we could use molecular techniques to design a CRD-BP complementary DNA that would generate an "epitope-tagged" CRD-BP. We could express the tagged CRD-BP in cells and then purify the CRD-BP in a single affinity step that exploits the tag to separate CRD-BP from all the other cell proteins.